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Impact of a chronic waterborne exposure to polystyrene nanoplastics on the gilthead seabream (*Sparus aurata*): combining traditional and multi-omics approaches

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Abstract

Recirculated aquaculture systems (RAS) represent valuable assets for meeting the growing seafood demand in a sustainable way. However, nanoplastic (NP <1000 nm, respectively) contamination still poses a risk in such systems. This study aimed to elucidate the effect of prolonged waterborne exposures to polystyrene nanoplastics (PSNPs) on one of the most commercially important fish species of Mediterranean aquaculture, the gilthead seabream (*Sparus aurata*). Juvenile fish were exposed to an environmentally relevant and a spiked concentration of PSNPs (100 µg/L and 1000 µg/L, respectively) over 28 days, and a series of traditional (*i.e.* morphometrics, haematology, histology) and omics (*i.e.* metabolomics, microbiome sequencing) approaches were employed to obtain a comprehensive understanding of the toxicity of PSNPs in *S. aurata*. The present results indicate that the studied experimental conditions did not impact morphometrics or haematological profile, nor did it cause histopathological lesions. However, waterborne PSNPs induced changes in the microbiome, significantly decreasing the Shannon's diversity index in fish exposed to high concentrations of PSNPs and altering the relative abundance of microbiota at the family level. Moreover, the metabolomic profile in the plasma of exposed *S. aurata* was altered in a dose-specific manner, with glutamate, proline, glycerol and formate levels being significantly up-regulated under spiked concentrations of PSNPs, and valine being significantly lower in individuals exposed to environmentally relevant concentrations. Overall, the results suggest that PSNPs might have potential repercussions particularly on the energy metabolism and overall performance of fish in the long term, affecting the production of the aquaculture industry.

Environmental Implication

Polystyrene nanoplastics (PSNPs) are one of the most common polymers in aquatic systems, whereas *Sparus aurata* is one of the most economically important fish species in the Mediterranean. The results indicate that PSNPs accumulate in the different organs of this species at different rates. Analysed muscle samples contained PSNPs, representing additional exposure routes to humans through the consumption of contaminated fish. In addition, the exposure led to alterations in the microbiome and metabolome of the gilthead seabream, which may have repercussions on its health status, affecting its growth, and having deleterious consequences on the performance of the aquaculture industry.

Keywords:

Fish welfare; metabolomics; microbiome; haematology; aquaculture

Introduction:

Global food security, an essential component to ensuring human health worldwide, faces a plethora of challenges. These include a variety of interconnected anthropogenic threats, such as climate change, overexploitation of natural resources and biodiversity loss, and environmental pollution, otherwise referred to as the triple planetary crisis. Today, plastic pollution has become a major concern to the scientific community due to the ever-increasing production and use of these materials and the poor management of plastic waste. Particularly, micro (<5 mm; Galgani et al., 2013) and nano (<1000 nm; Gigault et al., 2018) plastics (MPs and NPs) have gained attention and are now widely recognised as contaminants of emerging concern, being ubiquitous in both terrestrial and aquatic environments (Kumar et al., 2020; Llorca et al., 2021; Rosso et al., 2023; Ter Halle et al., 2017). Nanoplastics (NPs) can be classified into two categories. On one hand, primary NPs are directly manufactured nano-sized and are commonly used in personal care products and flame retardants (Gonçalves & Bebianno, 2021). On the other hand, secondary NPs are the result of the biotic and abiotic degradation of larger plastic objects (El Hadri et al., 2020). Therefore, with an increase in plastic production and discard (Yan et al., 2024), a rapid increase in the quantity of NPs in the environment is expected. These compounds have been detected and quantified in a large variety of environmental matrices (*e.g.* drinking water, throughout the oceanic water column, alpine snow), with polystyrene (PS) and polyethylene (PE) being the most commonly found polymers (Reviewed by Cai et al., 2021; Llorca et al., 2021).

Aquaculture, consisting of farming aquatic organisms, represents an invaluable asset in protecting biodiversity and safeguarding global food security (Yadav et al., 2024), if done sustainably, particularly in the current scenario of increasing world population (Gu et al., 2021). In particular, the use of recirculated aquaculture systems (RAS) offers the possibility to virtually isolate the farmed organisms, both sheltering them from environmental variables and minimising the impact of the practice on surrounding ecosystems. RAS protect the cultured species from climatic events, predators and fluctuations in environmental parameters (*e.g.* temperature, nitrogenous compounds, pathogens, pH; Ahmed & Turchini, 2021; Bregnballe, 2022; Summerfelt & Vinci, 2008). However, it has been demonstrated that NPs are still present in such systems, suggesting that they originate both from external (*i.e.* inlet water) and internal (*e.g.* bio-beads) sources (Blonç et al., 2023). Therefore, it is clear that aquaculture species, whether farmed in open or closed systems, are continuously exposed to these emerging contaminants.

The gilthead seabream (*Sparus aurata*) is one of the most economically important cultured fish species in the Mediterranean area (FAO, 2021, 2022; Mhalhel et al., 2023), and the presence of contaminants in the water, such as NPs, might have negative repercussions on the viability of the production by affecting reproduction and growth, amongst others, potentially affecting millions of people. Thus, it is of utmost importance to thoroughly investigate the effect that NPs might have on this species to better understand the implications of NP pollution for the aquaculture sector and for global food security.

Current research on the impact of NPs on fish has mainly focused on short-term, or acute, exposures (Barría et al., 2020), leaving only a few to investigate the effects of prolonged, or chronic, exposures (Brandts et al., 2021; Marana et al., 2022). Most of these chronic studies have researched the impact of NPs in model organisms, such as medaka (*Oryzias latipes*; Zhou et al., 2022; Zhou et al., 2023), zebrafish (*Danio rerio*; Sarasamma et al., 2020), and goldfish (*Carassius auratus;* Brandts et al., 2022). The results indicate that NPs may alter the lipid metabolism of fish, hindering their ability to efficiently allocate their energetic resources, affecting their reproduction, development (Brandts et al., 2022; Lin et al., 2023; Zhou et al., 2023), and behaviour (Sarasamma et al., 2020). Furthermore, it has been reported that NPs are internalised by aquatic organisms, accumulate in different tissues at differing rates, and travel through the food chain, reaching top-consumers by trophic transfer (He et al., 2022; Mattsson et al., 2017). Moreover, NPs have been described to significantly disturb the metabolome of *C. auratus* and *Carassius carassius* (Mattsson et al., 2015; Shi et al., 2021) and the gut microbiome of *D. rerio, O, latipes,* and *Sebastes schlegelii*, amongst others (Sun et al., 2023; Yu et al., 2022; Y. Zhou, Gui, et al., 2023).

However, the effect of NPs in aquatic organisms appears as highly dependent on the organisms' characteristics (*e.g.* species, life stage, sex), as well as the physio-chemical properties of the contaminant (*e.g.* polymer type, size, shape, purity), and the type of exposure (*e.g.* duration, administration route, co-contaminants; reviewed by Barría et al., 2020 and Gong et al., 2023). Due to the considerable quantity of factors influencing the toxicity of NPs, it is critical to refrain from making broad generalizations regarding the impact of nanoplastics on aquatic ecosystems, as doing so could undermine credibility with both the scientific community and policymakers.

Given the inherent intricacies of NPs toxicity, it is essential to combine a variety of analytical methods when investigating the impacts of NP pollution. Indeed, single-omics approaches, for instance, are not sufficient to understand the effect of NPs in living organisms (Bhagat et al., 2022), and must be coupled with additional assays in order to avoid drawing inaccurate conclusions.

To the best of the authors knowledge, the present study is the first to investigate the impact of a chronic waterborne exposure to PSNPs in a commercially important marine fish, in this case *S. aurata*. To this end, a multidisciplinary approach was employed, integrating traditional assays (*e.g*. less energy-, technologically, and economically demanding) and omics technologies (*e.g.* higher throughput and result yield) to acquire a comprehensive understanding of how this compound may affect the overall health and welfare status of this species, and its potential impact on the aquaculture sector and, ultimately, global food security. This study analysed retention rates of PSNPs, and the potential alterations in the morphometrics, histology, haematological profile, metabolome, and microbiome of the gilthead seabream induced by a 28-day exposure to waterborne PSNPs. These contaminants were selected given their omnipresence

in the environment, their commercial availability, and the frequency of studies investigating this specific polymer, therefore providing a substantial amount of data and scientific publications to build upon and compare the results of the present study.

Materials and methods:

Experimental animals housing and husbandry:
Juvenile *S. aurata* (N = 60; 10.35 ± 11.53 cm total length, and 13.69 ± 5.73 g total weight) were purchased from AVRAMAR (Castellón, Spain). The fish were held for three weeks in the AQUAB facilities at the Autonomous University of Barcelona, in 1000L tank containing aerated artificial sea water (ASW) at 35 ppt (Sea salt, Aquaforest) working under recirculation settings, and fed twice a day 2 % of the total biomass as a maintenance diet. Fish were then transferred into 20 L glass tanks of ASW, each containing 5 fish, for acclimation to the experimental conditions. Water parameters were checked daily to ensure water quality was optimal for the tested organism, and water changes were performed when necessary.

Experimental conditions:
Following the acclimation period, the fish were subjected to a 28-day (OECD, 2013) waterborne exposure to 40 nm PSNPs under three distinct experimental groups, namely, the control group (Ctrl: 0 µg/L), the low concentration group (LC: 100 µg/L PSNPs), being an environmentally relevant concentration, and the high concentration group (HC: 1000 µg/L PSNPs), representing a spiked concentration, 10x greater than those found in the environment. Each of the three experimental groups was represented by quadruplicated tanks.
To reach the required PSNPs concentration in the experimental tanks, a small volume of a stock PSNPs solution was dissolved in 1L of tank water, which was then added back to the tank. The water quality parameters were controlled on a daily basis, and 75% of the water was changed every 48h to prevent the accumulation of metabolites, ensuring optimal conditions for the experimental subjects during the trial. Following the water changes, fresh PSNPs were addedto maintain the desired concentration throughout the experimental period. Any individual displaying evident signs of distress was humanely euthanized through an overdose of tricaine methane sulfonate (MS-222; 300 mg/L, buffered with sodium bicarbonate at a 1:2 ratio) to prevent the unnecessary suffering of the animals.

Sampling:
Following the 28-day exposure to PSNPs, fish were individually euthanised by immersion in an MS-222 bath (300 mg/L) buffered with sodium bicarbonate ($NaHCO_3$; 600 mg/L), after which, death was confirmed through spinal rupture. Once the fish had reached stage 4 of anaesthesia (*i.e.* surgical anaesthesia), individuals were measured and weighed, and blood was immediately extracted using heparinised syringes (25G needles), mixed with lithium heparin (Deltalab, Spain) at a 25:1000 heparin to blood ratio, and preserved in dark conditions at 4 ºC until further processing and analysis. Blood samples were divided into three aliquots. One aliquot was used to obtain plasma through centrifugation (2000 *g*, 4 ºC, 10 min), another for semi-quantification of nanoplastics and the last one for a genotoxicity assay. Fish organs, namely, liver, gut, brain, muscle, and gills were collected under sterile conditions, and immediately snap frozen in liquid nitrogen. The samples destined for NPs semi-quantification were stored in crystal vials (Agilent Technologies). All tissue samples were then stored at -80 ºC until further processing and analysis.

Fulton's condition factor:

Total length (L, cm) and total weight (W, g) were used to determine the Fulton's condition factor (K_c) through the following equation: $K_c = 100 * \frac{W}{L^3}$.

Polystyrene nanoplastics characterisation:

Polystyrene nanoplastics of 40 nm (Bangs Laboratories) were characterized in different media (*i.e.* Ultrapure-MiliQ water and ASW) by means of dynamic light scattering (DLS; Zetasizer Pro, Malvern Instruments) to obtain particle size and polydispersity index (PDI). To this end, serial dilutions of the stock solution (100 mg/mL) were performed to obtain the experimental concentrations (*i.e.* 100 µg/L and 100 µg/L) and measurements were run 5 times at 25ºC at time 0h, 24h and 48h. A PDI value over 0.2 was considered an indicator of particle aggregation.

Polystyrene nanoplastics extraction and semi-quantification:

PSNPs were extracted from tissue samples and purified. In summary, the samples were digested with KOH (10 %) at 60 ºC, for 6 h, and then rested at room temperature overnight. Subsequently, the samples were filtered through 0.7 µm fiberglass filters where the residual organic material present was further digested at room temperature with HNO_3 at 20 % over 1 h. Following this, the filters were cleaned with water and dried overnight at 60 ºC. PSNPs were extracted using ultrasonic-assisted extraction with 10 mL of toluene for 10 min and the supernatant was transferred to crystal vials. This procedure was repeated twice again. The final extract was then evaporated with a nitrogen stream in order to concentrate the samples around 1.5 mL. The extracts were then vortexed and transferred to Liquid Chromatography vials and evaporated again to 1.5 mL. NPs were analysed by Liquid Chromatography coupled to High-Resolution Mass Spectrometry (HRMS) in an Acquity Liquid Chromatography (Waters, Milford) chromatographic system equipped with an advanced polymer chromatography column (Acquity APC XT45 1.7 µm) in isocratic conditions with toluene. The system was coupled to a Q-Exactive (Thermo Fisher Scientific) hybrid quadrupole-Orbitrap mass spectrometer, equipped with an atmospheric pressure photoionization source working in negative conditions. Data acquisition was performed in full scan mode (m/z 500-3000) with a resolution of 17,500 FWHM. Instrumental analysis contamination originating from the system was controlled by randomly injecting solvent blanks of toluene between samples. Moreover, exposure blanks were run on the system. Instrumental analysis, quality assurance and quality control were performed as described in (Llorca et al., 2021).

NMR Metabolomics in plasma:

Macromolecular components (>500KDa) were filtered from plasma using Amicon Ultra-0.5 Centrifugal FilterTubes. First, the filters were thoroughly washed with Mili-Q water and centrifuged for 5 minutes at 13793 *g* a total of four times to remove any potential traces of glycerol preservative. Once the filters were properly rinsed, the plasma samples were immediately centrifuged in the filter tubes at 13000 *g* for 30 minutes at 4 ºC. 200 µL of the obtained filtrate were then transferred to individual Nuclear Magnetic Resonance (NMR) tubes where 300 µL of the internal standard solution (*i.e.* 0.2 mM DSS in D_2O) was added, for a total volume of 500 µL and a final DSS concentration of 0.12 mM in each tube. ^{1}H NMR spectra were then measured in a Bruker 600-MHz AVANCE III NMR spectrometer equipped with a triple channel probe TXI 600 S3 H-C/N-D 05-Z-BTO (Bruker Biospin, Rheinstetten, Germany), operating at a ^{1}H frequency of 600.13 MHz and 298 K with a 5 min temperature equilibration time for each sample. The standard 1D ^{1}H-nuclear

Overhauser effect spectroscopy (1D-NOESY) pulse sequence from the Bruker library was used (noesygppr1d). The relevant parameters applied were: mixing time: 100 ms (d8), recovery delay: 2 s (d1), 90◦ pulse: 8.95 µs (p1), spectral width: 7211.539 Hz, spectral size: 32 k, number of scans 512 and acquisition time: 2.27 s. For metabolite identification and quantification data processing and analysis Spectra were processed and analysed through the Chenomx 8.0 profiler software (Chenomx Inc., Edmonton, Canada). This software contains tools for automatic phase, baseline correction (spline), automatic shim correction, reference calibration and a library of metabolites for spectral profiling. The concentration of each metabolite identified was determined based on the standard internal reference DSS (concentration 0.12 mmol/L). The obtained data were processed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA.). Two sets of statistical analyses were performed. For identified metabolites, non-parametric Kruskal-Wallis tests were undertaken, followed by a Wilcoxon test with Bonferroni correction. Furthermore, a principal component analysis (PCA) was performed to determine how many factors could separate the different metabolites, and a discriminant analysis was run to evaluate the components' capacity to differentiate the experimental groups. For non-identified metabolites, a qualitative assessment was performed by applying Chi-Squared tests or likelihood-ratio tests, as suitable. The *p*-value to reject null hypothesis was set at 0.05.

Blood analysis:

The haematological analyses were performed by inserting 90 µL of fresh heparinised blood in the automated haematological analyser XN-1000V (SYSMEX) working with an adapted version of the bird blood analysis software. Leukocyte and erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and thrombocyte count were determined.

To evaluate Erythrocyte Nuclear Abnormalities (ENA) blood smears were conducted with a drop of fresh blood on a microscope slide. These were coloured with a modified Romanowsky dye and observed under an optical microscope (100X magnification) for erythrocyte differentiation. 1000 erythrocytes in each sample were classified into two main classes, namely normal and abnormal red blood cells, with the latter being further subclassified according to the anomalies they presented (*i.e.* micronucleus, lobed nucleus, segmented nucleus and reniform nucleus), as previously described (Pacheco & Santos, 1996). Erythrocytes with intact membranes were exclusively selected for the analysis. The results are expressed as a frequency of ENAs per 1000 cells.

Histological analyses:

Gut, liver and gill samples were fixed in phosphate-buffered saline at pH 7.2 containing formalin (10 %). Subsequently, the preparations were washed in distilled water, dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Sections (6 µm) were cut and mounted on gelatinized slides using a rotary microtome. Sections were then rehydrated in distilled water and stained with hematoxylin/eosin (H&E) Giemsa or periodic acid-Schiff (PAS). Prepared slides were examined and photographed (Jenoptik ProgRes CT5) under a light microscope (Nikon eclipse Ci-L).

Microbiome analyses:

Genomic DNA was extracted from whole gut samples using the Power Soil Pro kit (Qiagen). Libraries were prepared for the sequencing of the v3-v4 region of the 16S gene. 12.5 ng of extracted DNA was amplified with 16S primers (Forward 5'-CCTACGGGNGGCWGCAG-

3' and Reverse 5'-GACTACHVGGGTATCTAATCC-3) equipped with the Illumina adapters. The PCR consisted of a 3-min hold at 95ºC, followed by a 30 s at 95 ºC – 30 s at 55 ºC – 30 s at 72 ºC cycle, repeated 25 times, and a final 5-min hold at 72 ºC. Following a magnetic bead-assisted-clean-up the corresponding indices were added to the Nextera® XT DNA Index (96 indices, 384 samples) FC-131-1002 (Illumina), and purified with magnetic beads. After each PCR the quality of the process was verified with a LabChip Bioanalyser (Agilent Technologies Inc.). Once all samples were obtained, these were multiplexed by mixing equimolar concentrations of each sample. All samples were sequenced in the MiSeq employing the MiSeq® Reagent Micro (500 cycles).
The sequencing reads were processed using USEARCH v.11 (R. C. Edgar, 2010) following the pipeline recommended by the author. In brief, the reads were truncated at the first sequence with a quality score (Q) below 30; the reads were then merged, and the primer binding sequences were stripped. The merged reads were quality-filtered and dereplicated to obtain unique sequences using the default setting. Denoising (*i.e.* predicting the true biological sequence (Amplicon Sequencing Variant - ASVs) from these unique sequences) was performed. The chimeric and singletons (*i.e.* sequences that appear below eight reads in all samples) were removed in the denoising process. Merged reads were mapped to ASVs to obtain the read counts for each sample. The taxonomic classification for the obtained ASVs was predicted using the SINTAX classifier (R. Edgar, 2016) with the RDP training set v19 as the reference database. The ASVs representing non-bacteria taxa (*e.g.* chloroplasts, eukaryotes, and taxa of known kit contaminants) were removed. From the sequencing of 24 samples, a total raw pair reads of 2.28 million read pairs were obtained. Of these raw pairs, 20.41 M pairs (89.41%) were successfully merged, with the mean length of the merged reads being 461 bp. Most non-mergeable raw reads were discarded (106.4K or 4.65%) because no alignment was found. The reads were rarefied to 54,502 reads (minimum reads in all samples). No samples were discarded. Finally, 24 samples and a count table with 1,154 ASVs and median reads of 82,654 reads per sample were used in the downstream analysis. The rarefaction analyses are plotted in Suppl. 3.
Hill's number 0 (observed ASV richness (S), 1 (exponential Shannon's entropy, exp. H'), and 2 (inverse Simpson ($1/D_S$) were selected to express community alpha diversity. Rarefying reads before calculating the diversity metrics could bias the estimate, and using a measurement error model is encouraged (Willis, 2019). The diversity values after extrapolation (*i.e.* error modelling) were used as implemented in the R package (iNEXT; Hsieh et al., 2016). Beta diversity was assessed using various distance metrics, namely, Bray-Curtis, Sørensen-Dice, Unifrac, and weighted Unifrac dissimilarity. Difference in alpha diversity of gut microbiome between treatment group was tested using pair-wise Wilcoxon test, while the difference in microbiome composition was investigated using PERMANOVA on the distance matrix based on Bray-Curti's dissimilarity metric. The differentially abundant families in the gut microbiome between fish in the control and high groups were investigated using the linear discriminant analysis of effect size (LEfSe) method (Segata et al., 2011). The p-value to reject null hypotheses was set at 0.05.

Statistical analysis:
Unless otherwise described in specific subsections of the Materials and Methods section, all data was tested for normality and homoscedasticity and analysed with one-way ANOVA or its non-parametric equivalent, the Kruskal-Wallis test, when suitable. All these statistical analyses were performed with GraphPad Prism v8.0.1, and the threshold for significance was considered at p-value <0.05.

Results:

Characterisation of polystyrene nanoplastics and semi-quantification in fish tissues:

DLS characterisation of PSNPs revealed medium-dependent particle size (Table 1). PSNPs in ultrapure-MiliQ water showed less dispersion in size than those in ASW. A PDI value over 0.2, as observed in ASW, indicates that the particles in this medium aggregated, as reflected by the increased in size. Nonetheless, the characterised particles remain, in all cases, in the nanoscale.

Table 1: Characterisation of polystyrene nanoplastics (100 µg/L and 1000 µg/L) in ultrapure-MiliQ water and artificial salt water (ASW, 35 ppt) at time 0h, 24h and 48h. Measured parameters were size (nm) and polydispersity index (PDI). Expressed as mean ± standard deviation.

		PDI (IU)			**Size (nm)**		
MEDIUM	PSNPs concentration \ time	0h	24h	48h	0h	24h	48h
ASW (35PPT)	100 ug/L	0.03	0.68	0.05	512.8	536.4	585.1
	1000 ug/L	0.57	0.78	0.73	603.2	468.1	616.7
MILIQ H2O	100 ug/L	1.51	0.39	0.56	45.1	53.4	55.33
	1000 ug/L	0.1	0.23	0.33	54.6	49.9	48.89

Quantification of PSNPs in fish organs revealed the presence of this compound in all brain and gill samples. PSNPs were only detected in one muscle sample obtained from the LC group, whereas none were detected in any of the Ctrl nor HC muscle samples. The analysis determined that none of the analysed blood samples contained PSNPs. In the liver, the analysis indicated the presence of PSNPs only in 60% of LC and HC samples, whereas none were detected in any Ctrl sample (Table 2).

Table 2: Proportion of samples of *Sparus aurata* blood, brain, liver, gut, muscle, and gills where polystyrene nanoplastics were detected following a 28-day waterborne exposure under three experimental conditions (Ctrl = Control group, 0 µg/L; LC = Low concentration group, 100 µg/L; HC = High concentration group, 1000 µg/L).

	Blood	*Brain*	*Liver*	*Gut*	*Muscle*	*Gills*
Ctrl (0 µg/L)	0%	100%	0%	100%	0%	100%
LC (100 µg/L)	0%	100%	60%	100%	20%	100%
HC (100 µg/L)	0%	100%	60%	60%	0%	100%
All groups	0%	100%	40%	87%	7%	100%

Morphometrics:

No significant differences were observed in either total length, weight, hepatosomatic index nor Fulton's condition factor between treatments (Suppl. 1).

Blood parameters:

The results obtained from the automated haematological analyser indicate no significant differences in any of the investigated parameters following the chronic waterborne exposure to PSNPs (Suppl. 2). Similarly, the Erythrocyte Nuclear Anomalies (ENA) assay did not result in significant differences in the proportion of normal and abnormal erythrocyte nuclei between experimental groups (Suppl. 2).

Histological analyses:

The direct observation of the prepared samples of gill (Figure 1A), gut (Figure 1B) and liver (Figure 1C) did not evidence any histopathological effect of the experimental conditions in *S. aurata* juveniles. In no case were the samples distinguishable between groups, and no cases of hyperplasia, dysplasia, inflammation or degenerative processes were found.

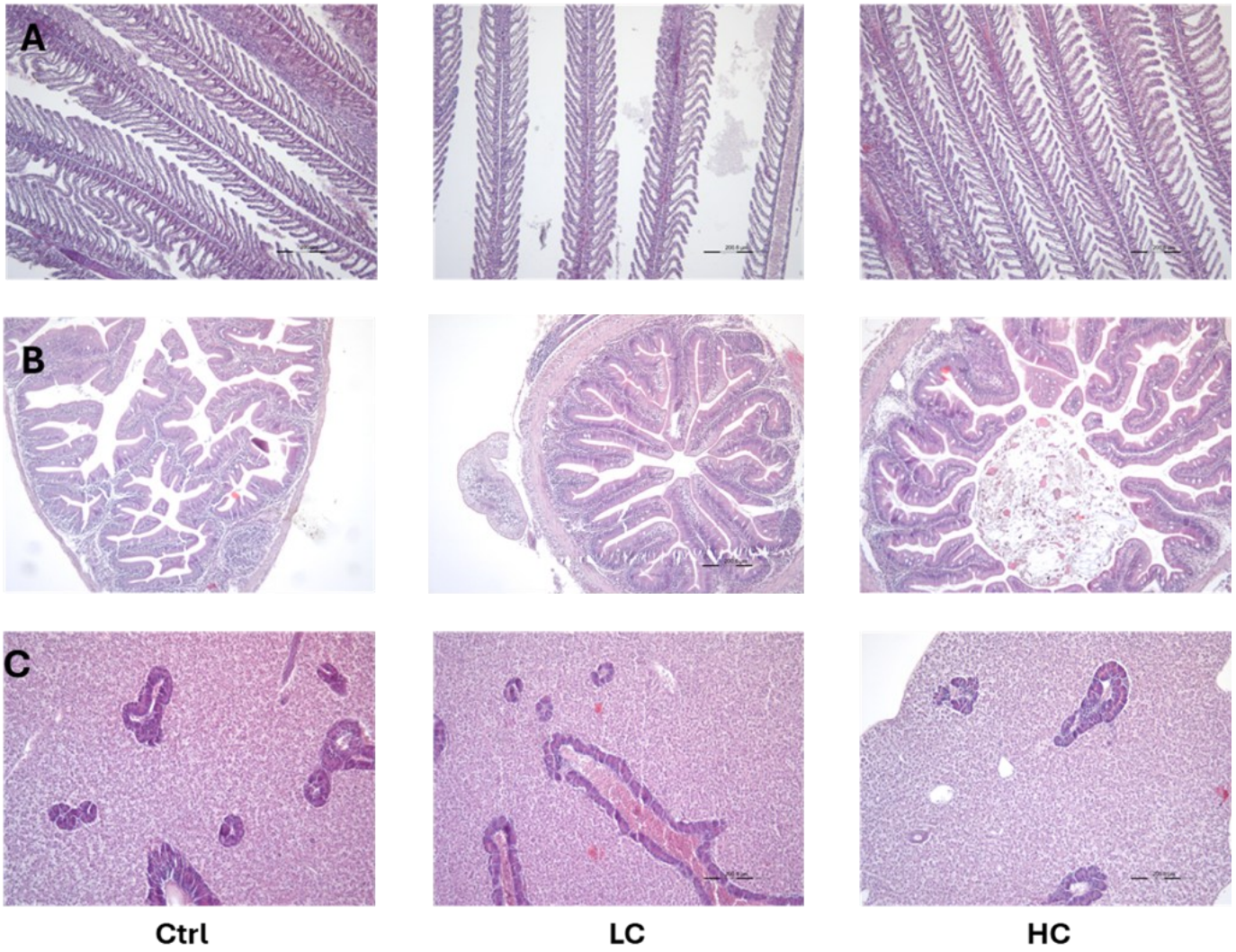


Figure 1: Histopathological observations of gills (A), gut (B), and liver (C) of juvenile gilthead seabream exposed to 0 µg/L (Ctrl), 100 µg/L (LC), and 1000 µg/L (HC) of waterborne polystyrene nanoplastics over a 28-day period.

Microbiome analysis:

Irrespective of experimental conditions, the most commonly identified bacterial phyla in the gut of *S. aurata* were *Proteobacteria* (*i.e.* family *Pseudomonadaceae*), followed by *Firmicutes* (*i.e*. families *Lactobacillaceae, Lachnospiraceae, Oscillospiraceae*, and *Clostridiaceae*),

Bacteroidetes (*i.e.* family *Prevolellaceae*), and *Actinobacteria* (*i.e.* family *Bifidobacteriaceae*). The analysis of the 16S amplicon sequencing data revealed significant differences in gut microbiome between experimental groups. Statistically significant differences arose in exponential Shannon's entropy, with HC displaying a significantly lower exponential Shannon's Diversity Index compared to the Ctrl and LC groups, but no differences were found between Ctrl and LC (Figure 2). No differences were found in either species richness or inverse Simpson's diversity index between any group. *Pseudomonadaceae* resulted as the most abundant family throughout the investigated samples, with a significantly greater representation in the HC group compared to the Ctrl. A visual representation of the differentially abundant taxa is shown in Figure 3, and detailed information on the taxa displaying statistically different abundances is given in Suppl. 4 Specifically, an increased relative abundance of *Firmicutes* was apparent in the LC group (order *Eubacteriales*), while a decrease was observed in the HC group (family *Lachnospiraceae*). The significant increase in the abundance of *Pseudomonodaceae* in the HC group was attributed to a proliferation of *Pseudomonas spp*. On the other hand, the significant increase in *Prevotellaceae* in the HC reflects the increase in abundance of *Prevotellamassilia spp.*

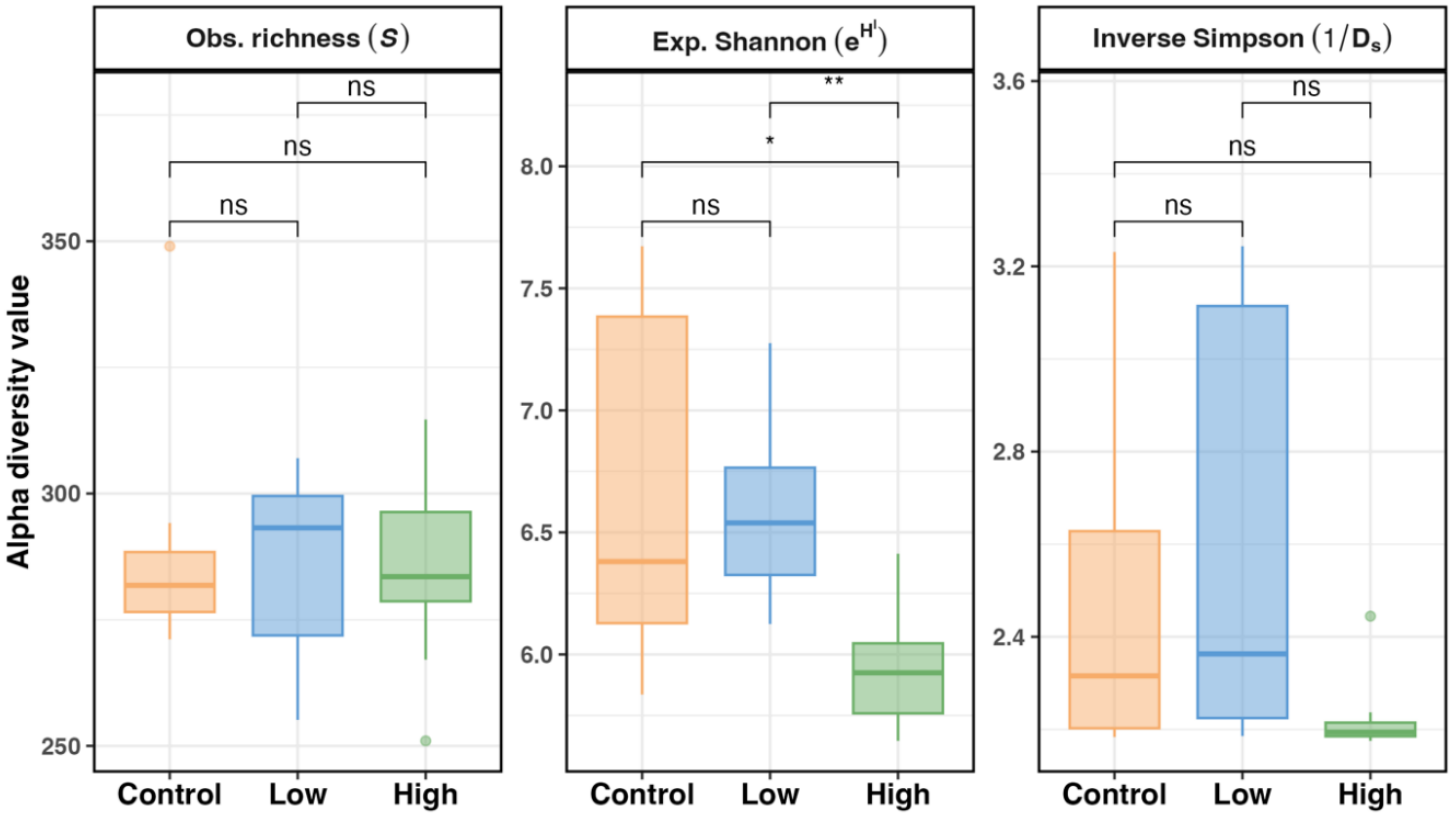


Figure 2: Gut microbiome alpha diversities of fish exposed to no (control), low (100 µg/L), and high (1000 µg/L) concentrations of polystyrene nanoplastics (ns = not significant, * = P-value < 0.05; ** = P-value < 0.01).

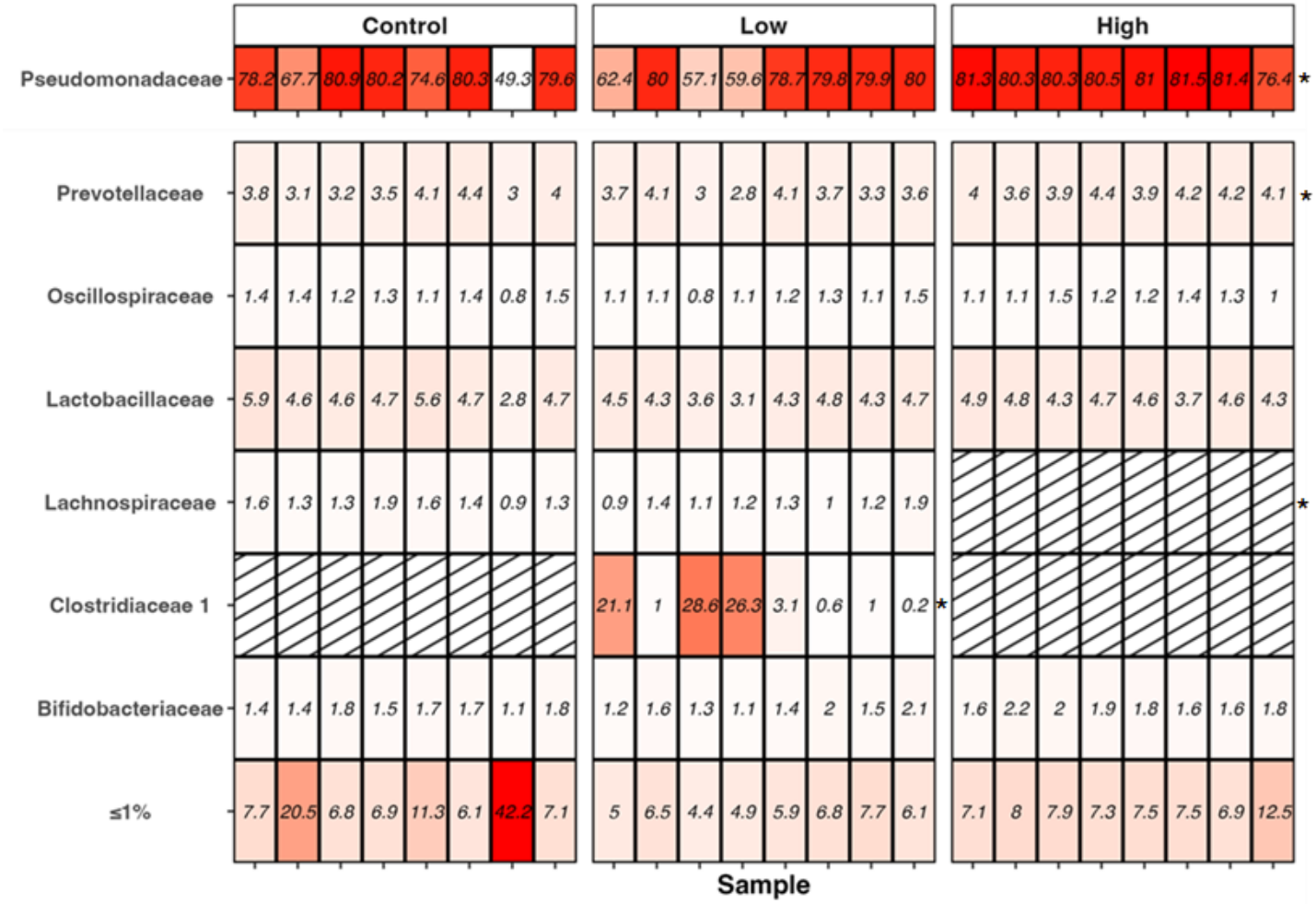


Figure 3: Heatmap of the family composition of bacteria community in the gut of fish exposed to polystyrene nanoplastics at 0 µg/L (control), low (100 µg/L), and high (1000 µg/L) concentrations. Stripped boxes indicated that the median relative abundance of the class in the experimental group is below 1%. Significant differences (P-value < 0.05) between one particular group and the other two treatments are indicated by * to the right of the concerned group.

NMR Metabolome analysis:

A total of 245 metabolites were detected, with 31 metabolites being identified and quantified (Spectra available in Suppl. 5). A PCA showed that 5 components explained over 86% of the differences observed between experimental groups (Suppl. 6). A discriminant analysis indicated that most samples were successfully separated by exposure condition, except for one LC sample which would have been classified as belonging to the Ctrl group (Suppl. 7). The statistical analyses of individual metabolites revealed significant differences between groups. HC group displayed significantly greater levels of glutamate, glycerol and formate when compared to the Ctrl group (Figure 5A, B and C). Additionally, proline levels resulted significantly higher in

the HC group compared to both the Ctrl and LC groups (Figure 5D). The LC groups exhibited significantly lower values of valine compared to the two other experimental groups (Figure 5E).

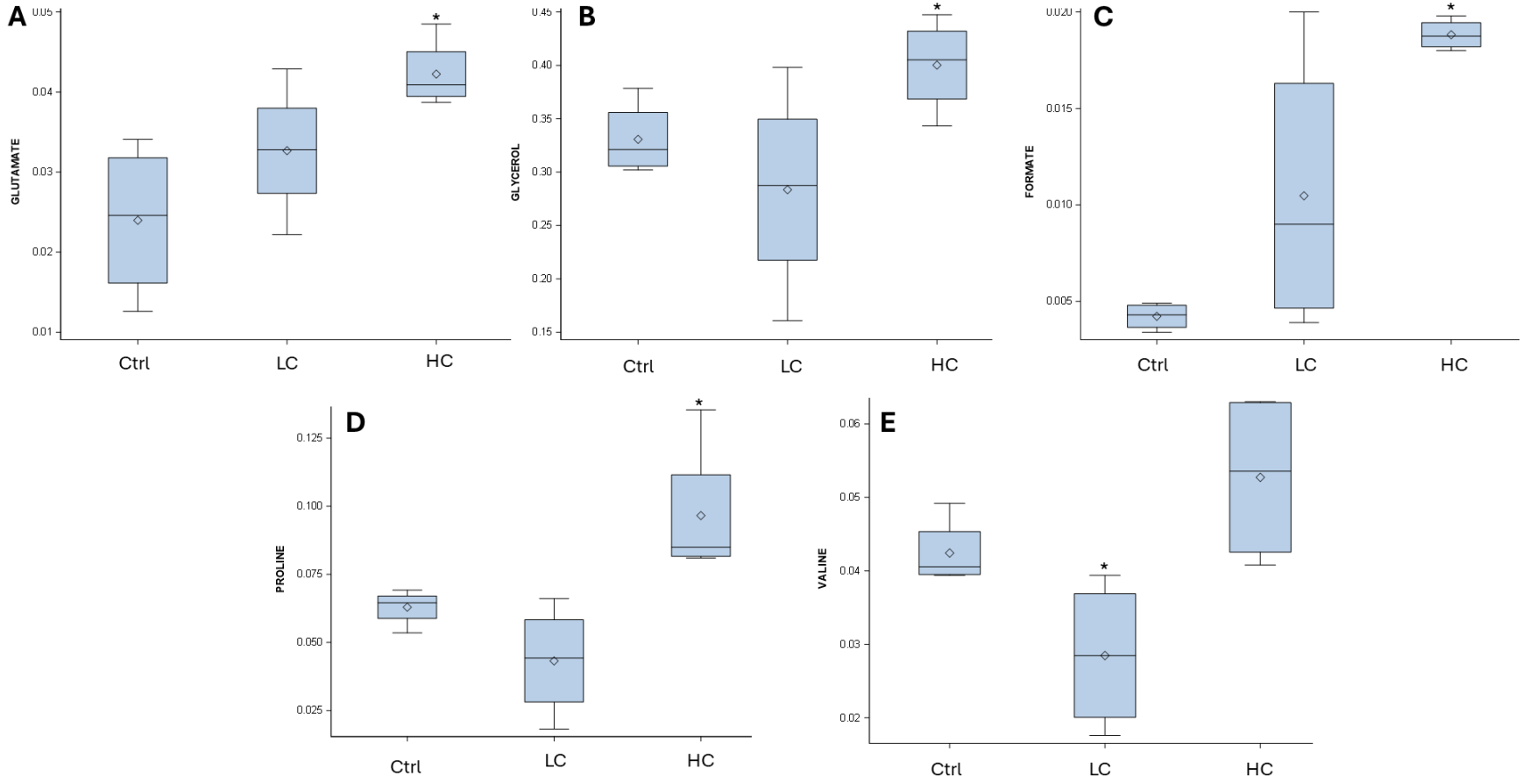


Figure 4: Variation of plasmatic levels of glutamate (A), glycerol (B), formate (C), proline (D), and valine (E) induced by a waterborne exposure to polystyrene nanoplastics at concentrations of 0 µg/L (control; Ctrl), low (100 µg/L; LC), and high (1000 µg/L; HC). Asterisks indicate significant differences with the control group (P-value < 0.05).

Discussion

The present study investigated the potential consequences of a 28-day waterborne exposure to PSNPs in *S. aurata* juveniles under laboratory conditions, including internalisation and retention of particles, as well as impact on health. The characterisation of PSNPs in the experimental medium revealed the aggregation of the particles due to the physical properties of both the media and the PSNPs particles (Boughbina-Portolés & Campíns-Falcó, 2024). The ultrapure water maintains the electrostatic repulsive forces which limit aggregation, whereas the ions in ASW present positive charges which interact with the negatively charged PSNPs surface, leading to a marked decrease in stability and resulting in aggregation (Tallec et al., 2019). The possibility of the particles agglomerating rather than aggregating was discarded given the inability of PSNPs to recover their original size following sonication (Boughbina-Portolés & Campíns-Falcó, 2024).

The qualitative analysis of NPs in fish tissues indicated that the brain and gills were the organs with highest affinity to PSNPs, with 100% of these samples containing PSNPs, including those of the Ctrl group. Similarly, 100% of gut samples from the Ctrl and LC group contained PSNPs traces, compared to only 60% of those in the HC group. In the liver, no PSNPs were detected in the Ctrl group, but these contaminants were identified in 60% of the exposed fish samples. The tissues that appeared to internalise the least PSNPs during the present experiment were muscle and blood, with 7% and 0% of all samples containing this pollutant, respectively (Table 1). This is in accordance with a number of past studies investigating the potential accumulation of PSNPs in fish and describing tissue-specific internalisation and retention rates. Sarasamma et al. (2020) reported that 70 nm fluorescent PSNPs were accumulated at a greater rate in zebrafish gonads than in any other organ, with gut, liver and brain samples containing similar PSNPs concentrations following a 30-day waterborne exposure. On the other hand, Hao et al. (2023) described significantly higher palladium-doped PSNPs levels in the gut of Nile tilapia (*Oreochromis niloticus*) compared to all other organs, irrespectively of size, following a 21-day exposure to 1 mg/L and a subsequent 7-day depuration period. In addition, the authors stated that muscle, gills and liver are more prone to accumulating smaller-sized NPs, with muscle samples displaying the highest quantified values of PSNPs. To the best of the authors' knowledge, no other study has investigated the accumulation of NPs in fish blood, and the absence of this compound in this tissue in the present study might be explained by the low retention time of contaminants in blood, which are rapidly translocated to the different organs. However, it is important to note that rather than reflecting their absolute absence in blood, the results indicate that the concentrations of PSNPs in blood were below detection limits. The detection of PSNPs in control samples reflects the already proven presence of this contaminant in Barcelona's urban and rural waters (Llorca et al., 2021; Vega-Herrera et al., 2022), to which the fish have been exposed since hatching, either at the RAS from where they originate, or in the laboratory facilities where the experiment was conducted. Moreover, PSNPs have also been proven to go airborne, and although no evidence is available for their presence in Barcelona's atmosphere, it is reasonable to assume their occurrence (Kjærgaard et al., 2024; Yao et al., 2022), In addition, the inconsistency in results between previously published studies, and with the present results, regarding the affinity of different fish tissues to PSNPs is likely explained by the differences in particle size and detection method, as well as experimental media (*i.e.* freshwater vs. saltwater) and the species of interest. The presence of NPs in the muscle of fish, particularly those of commercial importance, as demonstrated by previous research, as well as by the present study, is of particular concern, as it represents an additional exposure route to this pollutant for humans through the ingestion of contaminated food (Blonç et al., 2023; Brandts et al., 2022; Hao et al., 2023).

Traditional health indicators

In the present study, all Fulton's condition factor values resulted around 1.15, indicating a generally appropriate health status for the experimental subjects, with no significant impact of the exposure on this parameter (Suppl. 1). Similarly, no effect of treatment was observed in length, weight and HSI. This is consistent with previous research indicating a lack of significant alterations in these parameters following prolonged waterborne exposure to PSNPs in different species, such as *O. latipes* (Zhou, et al., 2023), *C. auratus* (Brandts et al., 2022), *Oreochromis niloticus* (Hao et al., 2023), and to a different polymer in *S. aurata* (Brandts et al., 2021). Nonetheless, these data should always be used complementary with other parameters, as drawing conclusions on the health status of a fish solely from morphometrics incurs the risk of drawing wrongful or inaccurate conclusions (Morton & Routledge, 2006). Morphometrics are common indicators of general health and fitness of individuals. In aquaculture, these data are used to infer on the performance of the produced organisms. Significant alterations in length, weight or Fulton's condition factor might indicate disrupted feeding behaviour, poor or insufficient feed quality and quantity, or the overallocation of energetic resources into survival (*e.g.* due to disease). Fulton's condition factor values in *S. aurata* should be greater than 1, with lower values indicating poor fish health or nutritional quality (Faggio et al., 2014). Similarly, the hepatosomatic index is commonly used as a proxy for the energetic status of fish, as well as an indicator for water contamination (Hauser-Davis et al., 2012).

The haematological analyses hereby performed did not show any significant differences in the investigated parameters between any of the experimental groups. This is in accordance with previously published findings describing a lack of haematological alterations in *C. auratus* following a 30-day waterborne exposure to 100 µg/L of 44 nm PSNPs (Brandts et al., 2022). On the other hand, previous studies investigating the impact of PS micro and NPs in water on *O. niloticus* have reported significant disturbances in their haematological profile in a size- and dose dependent manner. Such results might indicate, amongst other things, the occurrence of inflammatory mechanisms and the triggering of immune responses, as well as problems associated with oxygen transport. For instance, Sayed et al. (2024) found significantly lower values of RBC, PLT, HCT, and HGB in individuals exposed to 5 mg/L of 50 nm PSNPs. Similarly, Vineetha et al. (2024) described significantly lower levels of RBC, HCT, HGB and WBC, following a sub-chronic exposure to 0.1 mg/L, 1 mg/L and 10 mg/L of 100 nm PSNPs, but no differences in PLT. Moreover, Hamed et al. (2019) observed a significant reduction in RBC and HGB but no effect on HCT, WBC or PLT following an experimental exposure to 1 mg/L, 10 mg/L, and 100 mg/L of PSMPs and PSNPs (>100 nm).This indicates that even with the same species, different studies might obtain differing results, likely attributable to differences in analytical methods and particle size and shape. These three studies focusing on Nile tilapia all performed sub-chronic exposure lasting around two weeks. Therefore, differences between the present study and previously reported results might also arise from differences in exposure period,dosages, and from the inherent differences between freshwater and marine fishes.

The visual observation for ENAs revealed that the present experimental procedure did not induce significant genotoxic effects on *S. aurata* juveniles. In contrast, *C. auratus* exposed to similar concentrations of 44 nm PSNPs displayed significantly greater ENAs than control fish (Brandts et al., 2022). Such differences in results might also be attributed to the intrinsic differences between both organisms, being a marine and a freshwater species, respectively. Moreover, the differing

behaviour of NPs in salt and freshwater would lead to differences in particle agglomeration rate between both media, translating into differing impacts on aquatic organisms.

In the present study, the exposure to waterborne PSNPs did not incur any histopathological changes in the gut, gills or liver of the experimental subjects. Similarly, a study investigating the impact of waterborne PSNPs (100 nm) at concentrations ranging from 0.001 to 10 mg/L reported no histopathological effects on the gills, liver, gut and kidney of *D. rerio* (Lei et al., 2018). Moreover, Zhang et al. (2021) found no histopathology arising from a 30-day dietary exposure to 100 nm PSNPs. Nonetheless, a number of available scientific articles describe significant alterations in the morphology of different organs of a variety of species following both chronic and sub-chronic dietary and waterborne exposures, such as *O. niloticus* (Hao et al., 2023; Sayed et al., 2024; Vineetha et al., 2024), *C. auratus* (Abarghouei et al., 2021), and *S. schegelii* (Sun et al., 2023). It has also been demonstrated that PSNPs do not induce histological lesions in the different tissues of the same species at the same rate, with some organs remaining morphologically normal after prolonged exposures to these contaminants (Abarghouei et al., 2021; Zhou et al., 2023), and it is possible that some species are more susceptible to histological alterations from NPs than others.

Omics approach

Investigating the gut microbiome in fish can provide insightful information on their overall health status. Significant changes in relative abundance of different taxa, as observed here in treated groups compared to the control group, imply a disrupted community composition, and may indicate hindered physiological functions, reduced resistance to environmental pollutants and pathogens, and reduced overall fitness (Evariste et al., 2019; Xavier et al., 2024). Moreover, the dominance of *Pseudomonadaceae* family, which was even more pronounced in the HC group compared to the Ctrl, might be concerning, as members of this family are often associated with pathogenicity (Austin & Austin, 2012). The most abundant taxa found in the present study are in accordance with previous descriptions of the gut microbiome of *S. aurata* (Egerton et al., 2018; Estruch et al., 2015).

Overall, the present findings indicate a slight alteration of the gut microbiome in *S. aurata* exposed to PSNPs over a prolonged period. Although the information on the impact of waterborne nanoplastics on the gut microbiome of the gilthead seabream is scarce, numerous studies have investigated the effect of PSMP and PSNPs in other fish species, although the results are somewhat contradictory (Bao et al., 2024). For instance, Qiao et al. (2019) and Jin et al. (2018) both reported a significant increase in *Firmicutes* coupled with a significant decrease in the abundance of zebrafish gut *Proteobacteria* induced by waterborne exposure to PSMPs. The increase in *Firmicutes* was further corroborated after an exposure to PSNPs in *D. rerio* (Marana et al., 2022). On the other hand, Xie et al. (2021) described the opposite trend, with a significant reduction in the abundance of *Firmicutes* and an increase in *Proteobacteria* in the gut of the same fish species. Particle size and exposure duration seemed to play a major role in determining the effect of these contaminants in the gut microbiome and explain these inconsistent outcomes (Bao et al., 2024). In the present study a significant increase in *Pseudomonas* and *Prevotellamassilia* resulted from exposure to high concentrations of PSNPs. While the former is recognised as predominant in fish guts, with abrupt changes in the abundance of some species potentially linked to pathogenic outbreaks (Kačániová et al, 2017), no information is available on the role of the latter on fish intestinal health.

In this study, the major taxa were shared between experimental groups, and the observed differences are attributed to slightly shifted relative abundances of some taxa between control and treatment groups. However, the decrease in diversity as reflected by the significant drop in Shannon's Diversity value may indicate a certain degree of dysbiosis (Evariste et al., 2019). Disrupted gut microbiomes are often associated to morphological anomalies in the gastro-intestinal tract structure, such as inflammation (Jin et al., 2018), nonetheless, the gut histology results showed no evidence of structural alterations, implying that the decreased in the gut microbiome diversity did not induce an inflammatory response and might instead be intertwined with the alterations of the metabolomic profile of the experimental fish. Indeed, it has been suggested that in fish, as in humans, significant decreases in the diversity of intestinal microbiota might be reflected by changes in the metabolome (Qiao et al., 2019).

In the present study, the metabolomic profile of *S. aurata* juveniles resulted significantly altered, to some extent. The significant increase in plasmatic glutamate, a non-essential amino acid, observed in the present study may reflect disturbed metabolic processes in these fish. Glutamate is an abundant neurotransmitter in fish, and plays a major excitatory role (Trudeau et al., 1996, 2000). It appears as a versatile compound, involved in a variety of metabolic processes, playing a key function in gut health by modulating the structure of the gastrointestinal tract, and exhibiting antioxidant functions (Andersen et al., 2016). Moreover, glutamate is essential for the synthesis of gamma-aminobutyric acid (GABA), which acts as a inhibitory neurotransmitter (Park & Chu, 2022), and glutathione peroxidase, involved in antioxidant defences (Andersen et al., 2016). Therefore, the observed trend of glutamate hints towards inhibited GABA- or glutathione-synthesis-related pathways caused by the exposure to waterborne PSNPs. Ultimately, excessive accumulation of glutamate may lead to synapsis death (Aliakbarzadeh et al., 2023), causing serious neuronal damage and having deleterious consequences on the overall health and welfare of living organisms. Although, given the absence of quantitative analysis of GABA, the obtained data does not present any specific evidence, a previous study demonstrated that PSNPs interfere with GABA and GABA-related pathways in zebrafish embryos (Zhou et al., 2023). Further research is required to confirm this toxicity mechanism in *S. aurata* juveniles. Under some sorts of stress, such as starvation and physical injuries, glutamate levels have been described to drop, reflecting increased metabolic requirements (Andersen et al., 2016; Walker et al., 1996; Zhang et al., 2024). However, the response of this amino acid to MPs and NPs in fish displays, a high degree of dependence on a variety of parameters (*e.g.* polymer type, size and dose, and species). On one hand, zebrafish embryos exposed to PETNPs (70 nm) over a 24 h period, displayed significant decreases in glutamate (Bashirova et al., 2023), similar to what was observed when exposing zebrafish larvae to mask-derived MPs (1-20 μm) for 3 h (Hu et al., 2024), and adult marine medaka (*Oryzias melastigmas*) to PSMPs (10-200 μm) for 60 days (Ye et al., 2021). On the same note, Duan et al. (2020) described significant alterations in glutamate metabolism in zebrafish embryo exposed to 100 nm PSNPs over a 3-day lapse, stating that smaller particles had greater effects than their bigger counterparts. However, a 21-day waterborne exposure of adult Javanese medaka (*Oryzias javanicus*) to 5 μm PSMPs resulted in a significant increase in glutamate levels, in a clear dose-dependent manner (Usman et al., 2022), and *S. aurata* fed with PEMP-contaminated brine shrimp (*Artemia salina*; 10-20 μm MPs) over a 35-day period, displayed significantly higher glutamate levels (Jacob et al., 2021), as corroborated by the present results, further indicating that the toxicity of MPs and NPs, and the dynamics of glutamate response to these contaminants is dictated by a combination of polymer type, particle size and exposure type and duration.

Similarly to what was observed for glutamate, glycerol values were significantly greater in the HC group compared to the Ctrl. Glycerol is a metabolite resulting from the breakdown of triglycerides, and significant increases in this parameter might indicate strenuous physical activity or metabolic stress, reflecting a disturbed lipid metabolism, which is possibly the case here. A similar trend has been observed in the Australian bass (*Macquaria novemaculeata*) exposed to PSNPs through contaminated *Artemia spp*., which exhibited significant alterations in the levels of glycerol in a size- dependent manner, with 50nm PSNPs causing the most substantial upregulation (Afrose et al., 2024). Other instances of altered glycerol metabolism have been reported in *Sebastes schegelii* and *O. melastigmas* following sub-chronic and chronic exposure to waterborne and dietary PSNP and PSMPs, respectively (Sun et al., 2023; Ye et al., 2021).

Proline, another non-essential amino acid involved in the response against oxidative stress (Krishnan et al., 2008), was also significantly increased in *S. aurata* by the exposure to PSNPs at a concentration of 1000 µg/L. Significant alterations in proline metabolism have been described numerous times in fish exposed to PSMPs and PSNPs (*e.g*. Chu et al., 2024; Usman et al., 2022). In rare minnow (*Gobiocypris rarus*), a 14-day exposure to PSMPs and PSNPs (1 µm at 1 mg/L and 100 nm at 1 mg/L and 10 mg/L) led to a significant increase in the levels of this metabolite (Chu et al., 2024). Although such increases have also been observed in zebrafish embryos and adults (Qiao et al., 2019; Wan et al., 2019) exposed to PSMPs of sizes 5 and 50 µm, respectively, the effect of PS on proline metabolism has been suggested to be size-dependent, with smaller particles causing greater disruptions (Afrose et al., 2024; Ye et al., 2021).

On the contrary, valine levels in the plasma of juvenile *S. aurata* of the present study resulted significantly lower in individuals exposed to 100 µg/L, but not in those exposed to 1000 µg/L, once again indicating a dose-dependent effect. Information available in previously published literature supports this statement, given the variability in the response dynamics of this essential amino acid. Indeed, significant downregulations in measured valine concentrations have been reported in MPs-exposed marine medaka (Ye et al., 2021) and zebrafish embryos (Bashirova et al., 2023; Wan et al., 2019), as well as *S. schegelii* exposed to NPs (Sun et al., 2023). On the other hand, adult Javanese medaka and juvenile gilthead seabream exposed to PSMPs displayed significant increases in measured valine levels (Jacob et al., 2021; Usman et al., 2022).

To the best of the authors' knowledge, no study has described the dynamics of formate levels in the face of MPs and NPs in fish. In bacterial communities and mammals, this carboxylic acid has been described as an essential component of antioxidant defences (Thomas et al., 2016), and as playing a key role in intermediary and energy metabolism, being an important electron donor (Brosnan & Brosnan, 2016). Therefore, although the role of formate in fish is poorly understood, alterations in plasmatic levels of this metabolite might indicate disruptions in one-carbon and fatty acid metabolism, having serious implications for cellular function, energetic resources allocations and overall performance of individuals (Brosnan & Brosnan, 2016; Lamarre et al., 2013).

The discriminant analysis was able to successfully separate all samples from HC and Ctrl, whereas one sample from LC could have been considered as Ctrl, indicating that, under these experimental conditions, the high dose of PSNPs hereby employed had a greater impact on the metabolome of *S. aurata* juveniles than the low dose. Overall, the results obtained from the metabolomic assessment of *S. aurata* plasma revealed slight but significant alterations in various metabolic pathways, mostly involved in energetic metabolism and antioxidant responses. In fish, similar trends have been observed, although the specific changes vary greatly between species and experimental setups. Rather than large changes in a few metabolites, the present study found

that small variations in a wide range of metabolites drive the metabolomic response of *S. aurata* to waterborne PSNPs. This has been previously described in fish exposed to a variety of both micro- and nano-sized PS particles through different routes, with the most frequently reported changes being involved in energy metabolism and oxidative stress management (Marana et al., 2022; Mattsson et al., 2015; Wan et al., 2019; Zhao et al., 2021). Despite these significant changes in the metabolome, the lack of histopathological, haematological and morphological alterations in the exposed individuals indicates that the effect of a 28-day waterborne exposure to PSNPs in juvenile gilthead seabream is unlikely to have deleterious effects on the performance of individuals, particularly at environmentally relevant concentrations. However, additional metabolites displayed increasing or decreasing trends, indicating that, under more severe situations (*e.g.* longer duration) the effects might intensify, and growth and, potentially, survival might see themselves affected as a result of hindered energy metabolism (Cedervall et al., 2012; Mattsson et al., 2015; Sarasamma et al., 2020; Zhou et al., 2023; Zhou et al., 2023).

The inherent complexity of NPs toxicity and its high dependence on biotic and abiotic factors, combined with the ever-increasing quantity of studies investigating these contaminants results in an overwhelming quantity of data, often leading to differing or contradictory conclusions. Overall, the results obtained from the present study suggest a relatively low impact of the 28-day waterborne exposure to PSNPs in juvenile *S. aurata*, particularly when compared to shorter exposure periods in fish. This could reflect, to some extent, the ability of these fish to cope with this emerging contaminant after being exposed for some time, by the establishment of defence mechanisms. All the observed changes were detected through the use of omics technologies, further highlighting the importance of including these techniques in ecotoxicological studies to enhance the accuracy of the obtained results. The current study presents some limitations, which need to be acknowledged for the correct interpretation of the present results. These are related to the relatively low sensitivity of the NMR equipment (*i.e.* resulting in the low number of identified metabolites), and the inherent behaviour of the employed PSNPs in saltwater (*i.e.* resulting in some degree of aggregation, heterogeneity in the size of the particles, and a significantly greater mean size than that of the purchased NPs). Moreover, the degree of environmental relevance of the selected particles (*i.e.* NPs in the environment are mostly secondary, rather than primary, and of irregular shape), and the evident presence of PSNPs in control fish caused by their omnipresence in natural and urban water systems also limit the thoroughness of the experimental approach.

Nonetheless, this manuscript describes the first study to investigate the effects of NP pollution in one of the most economically important fish species in the Mediterranean area in such a comprehensive manner. It provides valuable data and lays the foundation for future studies to further investigate this issue, focusing on longer exposures, and looking into the possible adaptive response of fish to NPs, encouraging researchers to employ multidisciplinary and integrative multi-omics approaches.

General conclusions

The present study is the first to investigate the impact of PSNPs on juveniles of the commercially important *S. aurata* using a multidisciplinary approach, allowing for the obtention of a comprehensive understanding of the impact of this contaminant of emerging concern on this species, and the potential repercussions of nanoplastic pollution on the aquaculture industry. The results showed that waterborne PSNPs accumulated in multiple organs of juvenile *S. aurata* at different rates, particularly in the brain and gills which might have implications for the correct

development of the fish, potentially affecting their neuronal health, as well as their respiratory and osmoregulatory capacities. Moreover, although lower than expected, the internalisation and retention of PSNPs in the muscle of such a commercially important and gastronomically popular species represents an additional exposure route for humans to this contaminant. Surprisingly, expected histopathological lesions due to the presence of PSNPs in gills, muscle and liver, were not detected. In contrast, alterations were found in the gut microbiome, which might be linked, to some extent, to changes observed in the metabolomic profile of the plasma. Together, these differences might translate into a disrupted energy metabolism and development, hindered antioxidant defences, which may result in an overall reduction in fitness and performance. Overall, under the present experimental conditions, waterborne PSNPs did not significantly affect the performance of juvenile gilthead seabream, as evidenced by the absence of effects on morphometrics. Overall, results contrast strongly with those reported from acute exposures, indicating the potential occurrence of defence mechanisms put in place in front of a prolonged stress, although it is possible that a longer exposure period would cause a decreasing in the efficiency of said defences, and lead to a possible decrease in growth and health performance, as suggested by the metabolic indicators. Future research should, thus, focus on investigating the potential defence mechanisms developed by *S. aurata*, and delve on longer waterborne exposures to different environmentally relevant NPs using comprehensive, multi-omics approach to unveil the long-term implications of NPs contamination for the fish food production industry and global health.

Ethical approval:

This work has received approval for research ethics from the Ethical Review Committees of the Autonomous University of Barcelona and a proof/certificate of approval is available upon request.

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Bibliography:

Abarghouei, S., Hedayati, A., Raeisi, M., Hadavand, B. S., Rezaei, H., & Abed-Elmdoust, A. (2021). Size-dependent effects of microplastic on uptake, immune system, related gene expression and histopathology of goldfish (Carassius auratus). *Chemosphere*, *276*, 129977. https://doi.org/10.1016/j.chemosphere.2021.129977

Afrose, S., Tran, T. K. A., O'Connor, W., Pannerselvan, L., Carbery, M., Fielder, S., Subhaschandrabose, S., & Palanisami, T. (2024). Organ-specific distribution and size-dependent toxicity of polystyrene nanoplastics in Australian bass (Macquaria novemaculeata). *Environmental Pollution*, *341*, 122996. https://doi.org/10.1016/j.envpol.2023.122996

Ahmed, N., & Turchini, G. M. (2021). Recirculating aquaculture systems (RAS): Environmental solution and climate change adaptation. *Journal of Cleaner Production*, *297*, 126604. https://doi.org/10.1016/j.jclepro.2021.126604

Aliakbarzadeh, F., Rafiee, M., Khodagholi, F., Khorramizadeh, M. R., Manouchehri, H., Eslami, A., Sayehmiri, F., & Mohseni-Bandpei, A. (2023). Adverse effects of polystyrene nanoplastic and its binary mixtures with nonylphenol on zebrafish nervous system: From oxidative stress to impaired neurotransmitter system. *Environmental Pollution*, *317*, 120587. https://doi.org/10.1016/j.envpol.2022.120587

Andersen, S. M., Waagbø, R., & Espe, M. (2016). Functional amino acids in fish nutrition health and welfare. *Frontiers in Bioscience*, *8*(1), 757. https://doi.org/10.2741/757

Austin, B., & Austin, D. A. (2012). Pseudomonadaceae Representatives. In *Bacterial Fish Pathogens* (pp. 341–356). Springer Netherlands. https://doi.org/10.1007/978-94-007-4884-2_10

Bao, S., Yi, J., Xian, B., Rao, C., Xiang, D., Tang, W., & Fang, T. (2024). Global analysis of the adverse effects of micro- and nanoplastics on intestinal health and microbiota of fish. *Journal of Hazardous Materials*, *470*, 134157. https://doi.org/10.1016/j.jhazmat.2024.134157

Barría, C., Brandts, I., Tort, L., Oliveira, M., & Teles, M. (2020). Effect of nanoplastics on fish health and performance: A review. *Marine Pollution Bulletin*, *151*, 110791. https://doi.org/10.1016/j.marpolbul.2019.110791

Bashirova, N., Poppitz, D., Klüver, N., Scholz, S., Matysik, J., & Alia, A. (2023). A mechanistic understanding of the effects of polyethylene terephthalate nanoplastics in the zebrafish (Danio rerio) embryo. *Scientific Reports*, *13*(1), 1891. https://doi.org/10.1038/s41598-023-28712-y

Bhagat, J., Zang, L., Nishimura, N., & Shimada, Y. (2022). Application of omics approaches for assessing microplastic and nanoplastic toxicity in fish and seafood species. *TrAC Trends in Analytical Chemistry*, *154*, 116674. https://doi.org/10.1016/j.trac.2022.116674

Blonç, M., Husson, F., Llorca, M., Farré, M., Tort, L., Brandts, I., & Teles, M. (2023). Occurrence of micro- nanoplastics in a commercial recirculated aquaculture system and their translocation to cultured fish organs: A baseline study. *Journal of Hazardous Materials Advances*, *12*, 100381. https://doi.org/10.1016/j.hazadv.2023.100381

Boughbina-Portolés, A., & Campíns-Falcó, P. (2024). Assessing the size transformation of nanoplastics in natural water matrices. *Science of The Total Environment*, *953*, 176225. https://doi.org/10.1016/j.scitotenv.2024.176225

Brandts, I., Barría, C., Martins, M. A., Franco-Martínez, L., Barreto, A., Tvarijonaviciute, A., Tort, L., Oliveira, M., & Teles, M. (2021). Waterborne exposure of gilthead seabream (Sparus aurata) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels. *Journal of Hazardous Materials*, *403*, 123590. https://doi.org/10.1016/j.jhazmat.2020.123590

Brandts, I., Cánovas, M., Tvarijonaviciute, A., Llorca, M., Vega, A., Farré, M., Pastor, J., Roher, N., & Teles, M. (2022). Nanoplastics are bioaccumulated in fish liver and muscle and cause DNA damage after a chronic exposure. *Environmental Research*, *212*, 113433. https://doi.org/10.1016/j.envres.2022.113433

Bregnballe, J. (2022). *A guide to recirculation aquaculture – An introduction to the new environmentally friendly and highly productive closed fish farming systems.* FAO and Eurofish International Organisation. https://doi.org/10.4060/cc2390en

Brosnan, M. E., & Brosnan, J. T. (2016). Formate: The Neglected Member of One-Carbon Metabolism. *Annual Review of Nutrition*, *36*(1), 369–388. https://doi.org/10.1146/annurev-nutr-071715-050738

Cai, H., Xu, E. G., Du, F., Li, R., Liu, J., & Shi, H. (2021). Analysis of environmental nanoplastics: Progress and challenges. *Chemical Engineering Journal*, *410*, 128208. https://doi.org/10.1016/j.cej.2020.128208

Cedervall, T., Hansson, L.-A., Lard, M., Frohm, B., & Linse, S. (2012). Food Chain Transport of Nanoparticles Affects Behaviour and Fat Metabolism in Fish. *PLoS ONE*, *7*(2), e32254. https://doi.org/10.1371/journal.pone.0032254

Chu, T., Zhang, R., Guo, F., Zhu, M., Zan, S., & Yang, R. (2024). The toxicity of polystyrene micro- and nano-plastics on rare minnow (Gobiocypris rarus) varies with the particle size and concentration. *Aquatic Toxicology*, *269*, 106879. https://doi.org/10.1016/j.aquatox.2024.106879

Duan, Z., Duan, X., Zhao, S., Wang, X., Wang, J., Liu, Y., Peng, Y., Gong, Z., & Wang, L. (2020). Barrier function of zebrafish embryonic chorions against microplastics and nanoplastics and its impact on embryo development. *Journal of Hazardous Materials*, *395*, 122621. https://doi.org/10.1016/j.jhazmat.2020.122621

Edgar, R. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, *081257*.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.00873

El Hadri, H., Gigault, J., Maxit, B., Grassl, B., & Reynaud, S. (2020). Nanoplastic from mechanically degraded primary and secondary microplastics for environmental assessments. *NanoImpact*, *17*, 100206. https://doi.org/10.1016/j.impact.2019.100206

Estruch, G., Collado, M. C., Peñaranda, D. S., Tomás Vidal, A., Jover Cerdá, M., Pérez Martínez, G., & Martinez-Llorens, S. (2015). Impact of Fishmeal Replacement in Diets for Gilthead Sea Bream (Sparus aurata) on the Gastrointestinal Microbiota Determined by Pyrosequencing the 16S rRNA Gene. *PLOS ONE*, *10*(8), e0136389. https://doi.org/10.1371/journal.pone.0136389

Evariste, L., Barret, M., Mottier, A., Mouchet, F., Gauthier, L., & Pinelli, E. (2019). Gut microbiota of aquatic organisms: A key endpoint for ecotoxicological studies. *Environmental Pollution*, *248*, 989–999. https://doi.org/10.1016/j.envpol.2019.02.101

Faggio, C., Piccione, G., Marafioti, S., Arfuso, F., Fortino, G., & Fazio, F. (2014). Metabolic Response to Monthly Variations of Sparus aurata Reared in Mediterranean On-Shore Tanks . *Turkish Journal of Fisheries and Aquatic Sciences*, *14*(2). https://doi.org/10.4194/1303-2712-v14_2_28

FAO. (2021). *GLOBEFISH Highlights - A quarterly update on world seafood markets*. FAO. https://doi.org/10.4060/cb4129en

FAO. (2022). *The State of World Fisheries and Aquaculture: Towards Blue Transformation*.

Galgani, F., Hanke, G., Werner, S., & De Vrees, L. (2013). Marine litter within the European

Marine Strategy Framework Directive. *ICES Journal of Marine Science*, *70*(6), 1055–1064. https://doi.org/10.1093/icesjms/fst122

Gigault, J., Halle, A. ter, Baudrimont, M., Pascal, P.-Y., Gauffre, F., Phi, T.-L., El Hadri, H., Grassl, B., & Reynaud, S. (2018). Current opinion: What is a nanoplastic? *Environmental Pollution*, *235*, 1030–1034. https://doi.org/10.1016/j.envpol.2018.01.024

Gonçalves, J. M., & Bebianno, M. J. (2021). Nanoplastics impact on marine biota: A review. *Environmental Pollution*, *273*, 116426. https://doi.org/10.1016/j.envpol.2021.116426

Gong, H., Li, R., Li, F., Guo, X., Xu, L., Gan, L., Yan, M., & Wang, J. (2023). Toxicity of nanoplastics to aquatic organisms: Genotoxicity, cytotoxicity, individual level and beyond individual level. *Journal of Hazardous Materials*, *443*, 130266. https://doi.org/10.1016/j.jhazmat.2022.130266

Gu, D., Andreev, K., & E. Dupre, M. (2021). Major Trends in Population Growth Around the World. *China CDC Weekly*, *3*(28), 604–613. https://doi.org/10.46234/ccdcw2021.160

Hamed, M., Soliman, H. A. M., Osman, A. G. M., & Sayed, A. E.-D. H. (2019). Assessment the effect of exposure to microplastics in Nile Tilapia (Oreochromis niloticus) early juvenile: I. blood biomarkers. *Chemosphere*, *228*, 345–350. https://doi.org/10.1016/j.chemosphere.2019.04.153

Hao, T., Gao, Y., Li, Z.-C., Zhou, X.-X., & Yan, B. (2023). Size-Dependent Uptake and Depuration of Nanoplastics in Tilapia (*Oreochromis niloticus*) and Distinct Intestinal Impacts. *Environmental Science & Technology*, *57*(7), 2804–2812. https://doi.org/10.1021/acs.est.2c08059

Hauser-Davis, R. A., Lavandier, R. C., Bastos, F. F., Oliveira, T. F., Ribeiro, C. A. O., Ziolli, R. L., & de Campos, R. C. (2012). Alterations in Morphometric and Organosomatic Indices and Histopathological Analyses Indicative of Environmental Contamination in Mullet, Mugil liza, from Southeastern Brazil. *Bulletin of Environmental Contamination and Toxicology*, *89*(6), 1154–1160. https://doi.org/10.1007/s00128-012-0846-x

He, S., Chi, H.-Y., Li, C., Gao, Y., Li, Z.-C., Zhou, X.-X., & Yan, B. (2022). Distribution, bioaccumulation, and trophic transfer of palladium-doped nanoplastics in a constructed freshwater ecosystem. *Environmental Science: Nano*, *9*(4), 1353–1363. https://doi.org/10.1039/D1EN00940K

Hsieh, T. C., Ma, K. H., & Chao, A. (2016). iNEXT: an R package for rarefaction and extrapolation of species diversity (<scp>H</scp> ill numbers). *Methods in Ecology and Evolution*, *7*(12), 1451–1456. https://doi.org/10.1111/2041-210X.12613

Hu, F., Zhao, H., Ding, J., Jing, C., Zhang, W., & Chen, X. (2024). Uptake and toxicity of micro-/nanoplastics derived from naturally weathered disposable face masks in developing zebrafish: Impact of COVID-19 pandemic on aquatic life. *Environmental Pollution*, *343*, 123129. https://doi.org/10.1016/j.envpol.2023.123129

Jacob, H., Besson, M., Oberhaensli, F., Taylor, A., Gillet, B., Hughes, S., Melvin, S. D., Bustamante, P., Swarzenski, P. W., Lecchini, D., & Metian, M. (2021). A multifaceted assessment of the effects of polyethylene microplastics on juvenile gilthead seabreams (Sparus aurata). *Aquatic Toxicology*, *241*, 106004. https://doi.org/10.1016/j.aquatox.2021.106004

Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., & Fu, Z. (2018). Polystyrene microplastics induce

microbiota dysbiosis and inflammation in the gut of adult zebrafish. *Environmental Pollution*, *235*, 322–329. https://doi.org/10.1016/j.envpol.2017.12.088

Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Klūga, A., Tokár, M., Kluz, M. and Ivanišová, E., 2017. The antioxidant and antimicrobial activity of essential oils against Pseudomonas spp. isolated from fish. *Saudi Pharmaceutical Journal*, *25*(8), pp.1108-1116.

Kjærgaard, E. R., Hasager, F., Petters, S. S., Glasius, M., & Bilde, M. (2024). Bubble-mediated generation of airborne nanoplastic particles. *Environmental Science: Processes & Impacts*, *26*(7), 1216–1226. https://doi.org/10.1039/D4EM00124A

Krishnan, N., Dickman, M. B., & Becker, D. F. (2008). Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress. *Free Radical Biology and Medicine*, *44*(4), 671–681. https://doi.org/10.1016/j.freeradbiomed.2007.10.054

Kumar, M., Xiong, X., He, M., Tsang, D. C. W., Gupta, J., Khan, E., Harrad, S., Hou, D., Ok, Y. S., & Bolan, N. S. (2020). Microplastics as pollutants in agricultural soils. *Environmental Pollution*, *265*, 114980. https://doi.org/10.1016/j.envpol.2020.114980

Lamarre, S. G., Morrow, G., Macmillan, L., Brosnan, M. E., & Brosnan, J. T. (2013). Formate: an essential metabolite, a biomarker, or more? *Clinical Chemistry and Laboratory Medicine*, *51*(3). https://doi.org/10.1515/cclm-2012-0552

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *Science of The Total Environment*, *619–620*, 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103

Lin, X., Wang, Y., Yang, X., Watson, P., Yang, F., & Liu, H. (2023). Endocrine disrupting effect and reproductive toxicity of the separate exposure and co-exposure of nano-polystyrene and diethylstilbestrol to zebrafish. *Science of The Total Environment*, *865*, 161100. https://doi.org/10.1016/j.scitotenv.2022.161100

Llorca, M., Vega-Herrera, A., Schirinzi, G., Savva, K., Abad, E., & Farré, M. (2021). Screening of suspected micro(nano)plastics in the Ebro Delta (Mediterranean Sea). *Journal of Hazardous Materials*, *404*, 124022. https://doi.org/10.1016/j.jhazmat.2020.124022

Marana, M. H., Poulsen, R., Thormar, E. A., Clausen, C. G., Thit, A., Mathiessen, H., Jaafar, R., Korbut, R., Hansen, A. M. B., Hansen, M., Limborg, M. T., Syberg, K., & von Gersdorff Jørgensen, L. (2022). Plastic nanoparticles cause mild inflammation, disrupt metabolic pathways, change the gut microbiota and affect reproduction in zebrafish: A full generation multi-omics study. *Journal of Hazardous Materials*, *424*, 127705. https://doi.org/10.1016/j.jhazmat.2021.127705

Mattsson, K., Ekvall, M. T., Hansson, L.-A., Linse, S., Malmendal, A., & Cedervall, T. (2015). Altered Behavior, Physiology, and Metabolism in Fish Exposed to Polystyrene Nanoparticles. *Environmental Science & Technology*, *49*(1), 553–561. https://doi.org/10.1021/es5053655

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L.-A., & Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific Reports*, *7*(1), 11452. https://doi.org/10.1038/s41598-017-10813-0

Mhalhel, K., Levanti, M., Abbate, F., Laurà, R., Guerrera, M. C., Aragona, M., Porcino, C., Briglia, M., Germanà, A., & Montalbano, G. (2023). Review on Gilthead Seabream (Sparus aurata) Aquaculture: Life Cycle, Growth, Aquaculture Practices and Challenges. *Journal of Marine Science and Engineering*, *11*(10), 2008. https://doi.org/10.3390/jmse11102008

Morton, A., & Routledge, R. D. (2006). Fulton's Condition Factor: Is it a Valid Measure of Sea Lice Impact on Juvenile Salmon? *North American Journal of Fisheries Management*, *26*(1), 56–62. https://doi.org/10.1577/M05-068.1

OECD. (2013). *OECD Guidelines for the Testing of Chemicals: Fish, Early-life Stage Toxicity Test (210)*.

Pacheco, M., & Santos, M. A. (1996). Induction of micronuclei and nuclear abnormalities in the erythrocytes of Anguilla anguilla L. exposed either to cyclophosphamide or to bleached kraft pulp mill effluent. *Fresenius Environmental Bulletin*, *5*(11–12), 746–751.

Park, C. G., & Chu, M. K. (2022). Interictal plasma glutamate levels are elevated in individuals with episodic and chronic migraine. *Scientific Reports*, *12*(1), 6921. https://doi.org/10.1038/s41598-022-10883-9

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., & Lemos, B. (2019). Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Science of The Total Environment*, *662*, 246–253. https://doi.org/10.1016/j.scitotenv.2019.01.245

Rosso, B., Corami, F., Barbante, C., & Gambaro, A. (2023). Quantification and identification of airborne small microplastics (<100 µm) and other microlitter components in atmospheric aerosol via a novel elutriation and oleo-extraction method. *Environmental Pollution*, *318*, 120889. https://doi.org/10.1016/j.envpol.2022.120889

Sarasamma, S., Audira, G., Siregar, P., Malhotra, N., Lai, Y.-H., Liang, S.-T., Chen, J.-R., Chen, K. H.-C., & Hsiao, C.-D. (2020). Nanoplastics Cause Neurobehavioral Impairments, Reproductive and Oxidative Damages, and Biomarker Responses in Zebrafish: Throwing up Alarms of Wide Spread Health Risk of Exposure. *International Journal of Molecular Sciences*, *21*(4), 1410. https://doi.org/10.3390/ijms21041410

Sayed, A. E.-D. H., Emeish, W. F. A., Bakry, K. A., Al-Amgad, Z., Lee, J.-S., & Mansour, S. (2024). Polystyrene nanoplastic and engine oil synergistically intensify toxicity in Nile tilapia, Oreochromis niloticus. *BMC Veterinary Research*, *20*(1), 143. https://doi.org/10.1186/s12917-024-03987-z

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shi, W., Sun, S., Han, Y., Tang, Y., Zhou, W., Du, X., & Liu, G. (2021). Microplastics impair olfactory-mediated behaviors of goldfish Carassius auratus. *Journal of Hazardous Materials*, *409*, 125016. https://doi.org/10.1016/j.jhazmat.2020.125016

Summerfelt, S. T., & Vinci, B. J. (2008). Better management practices for recirculating aquaculture systems. In C. S. Tucker & J. A. Hargreaves (Eds.), *Environmental best management practices for aquaculture* (pp. 389–426). Wiley. https://doi.org/10.1002/9780813818672

Sun, X., Wang, X., Booth, A. M., Zhu, L., Sui, Q., Chen, B., Qu, K., & Xia, B. (2023). New

insights into the impact of polystyrene micro/nanoplastics on the nutritional quality of marine jacopever (Sebastes schlegelii). *Science of The Total Environment*, *903*, 166560. https://doi.org/10.1016/j.scitotenv.2023.166560

Tallec, K., Blard, O., González-Fernández, C., Brotons, G., Berchel, M., Soudant, P., Huvet, A., & Paul-Pont, I. (2019). Surface functionalization determines behavior of nanoplastic solutions in model aquatic environments. *Chemosphere*, *225*, 639–646. https://doi.org/10.1016/j.chemosphere.2019.03.077

Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., & Gigault, J. (2017). Nanoplastic in the North Atlantic Subtropical Gyre. *Environmental Science & Technology*, *51*(23), 13689–13697. https://doi.org/10.1021/acs.est.7b03667

Thomas, S. C., Alhasawi, A., Auger, C., Omri, A., & Appanna, V. D. (2016). The role of formate in combatting oxidative stress. *Antonie van Leeuwenhoek*, *109*(2), 263–271. https://doi.org/10.1007/s10482-015-0629-6

Trudeau, V. L., Sloley, B. D., Kah, O., Mons, N., Dulka, J. G., & Peter, R. E. (1996). Regulation of Growth Hormone Secretion by Amino Acid Neurotransmitters in the Goldfish (I): Inhibition byN-Methyl-d,l-aspartic Acid. *General and Comparative Endocrinology*, *103*(2), 129–137. https://doi.org/10.1006/gcen.1996.0103

Trudeau, V. L., Spanswick, D., Fraser, E. J., Larivière, K., Crump, D., Chiu, S., MacMillan, M., & Schulz, R. W. (2000). The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochemistry and Cell Biology*, *78*(3), 241–259. https://doi.org/10.1139/o99-075

Usman, S., Razis, A. F. A., Shaari, K., Azmai, M. N. A., Saad, M. Z., Isa, N. M., & Nazarudin, M. F. (2022). Polystyrene microplastics induce gut microbiome and metabolome changes in Javanese medaka fish (Oryzias javanicus Bleeker, 1854). *Toxicology Reports*, *9*, 1369–1379. https://doi.org/10.1016/j.toxrep.2022.05.001

Vega-Herrera, A., Llorca, M., Borrell-Diaz, X., Redondo-Hasselerharm, P. E., Abad, E., Villanueva, C. M., & Farré, M. (2022). Polymers of micro(nano) plastic in household tap water of the Barcelona Metropolitan Area. *Water Research*, *220*, 118645. https://doi.org/10.1016/j.watres.2022.118645

Vineetha, V. P., Suresh, K., & Pillai, D. (2024). Impact of sub-chronic polystyrene nanoplastics exposure on hematology, histology, and endoplasmic reticulum stress-related protein expression in Nile tilapia (Oreochromis niloticus). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *273*, 110982. https://doi.org/10.1016/j.cbpb.2024.110982

Walker, S. P., Keast, D., & McBride, S. (1996). Distribution of glutamine synthetase in the snapper (Pagrus auratus) and implications for the immune system. *Fish Physiology and Biochemistry*, *15*(3), 187–194. https://doi.org/10.1007/BF01875569

Wan, Z., Wang, C., Zhou, J., Shen, M., Wang, X., Fu, Z., & Jin, Y. (2019). Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. *Chemosphere*, *217*, 646–658. https://doi.org/10.1016/j.chemosphere.2018.11.070

Willis, A. D. (2019). Rarefaction, Alpha Diversity, and Statistics. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02407

Xavier, R., Severino, R., & Silva, S. M. (2024). Signatures of dysbiosis in fish microbiomes in

the context of aquaculture. *Reviews in Aquaculture*, *16*(2), 706–731. https://doi.org/10.1111/raq.12862

Xie, S., Zhou, A., Wei, T., Li, S., Yang, B., Xu, G., & Zou, J. (2021). Nanoplastics Induce More Serious Microbiota Dysbiosis and Inflammation in the Gut of Adult Zebrafish than Microplastics. *Bulletin of Environmental Contamination and Toxicology*, *107*(4), 640–650. https://doi.org/10.1007/s00128-021-03348-8

Yadav, N. K., Patel, A. B., Singh, S. K., Mehta, N. K., Anand, V., Lal, J., Dekari, D., & Devi, N. C. (2024). Climate change effects on aquaculture production and its sustainable management through climate-resilient adaptation strategies: a review. *Environmental Science and Pollution Research*, *31*(22), 31731–31751. https://doi.org/10.1007/s11356-024-33397-5

Yan, H., Cordier, M., & Uehara, T. (2024). Future Projections of Global Plastic Pollution: Scenario Analyses and Policy Implications. *Sustainability*, *16*(2), 643. https://doi.org/10.3390/su16020643

Yao, Y., Glamoclija, M., Murphy, A., & Gao, Y. (2022). Characterization of microplastics in indoor and ambient air in northern New Jersey. *Environmental Research*, *207*, 112142. https://doi.org/10.1016/j.envres.2021.112142

Ye, G., Zhang, X., Liu, X., Liao, X., Zhang, H., Yan, C., Lin, Y., & Huang, Q. (2021). Polystyrene microplastics induce metabolic disturbances in marine medaka (Oryzias melastigmas) liver. *Science of The Total Environment*, *782*, 146885. https://doi.org/10.1016/j.scitotenv.2021.146885

Yu, Z., Zhang, L., Huang, Q., Dong, S., Wang, X., & Yan, C. (2022). Combined effects of micro-/nano-plastics and oxytetracycline on the intestinal histopathology and microbiome in zebrafish (Danio rerio). *Science of The Total Environment*, *843*, 156917. https://doi.org/10.1016/j.scitotenv.2022.156917

Zhang, L., García-Pérez, P., Muñoz-Palazon, B., Gonzalez-Martinez, A., Lucini, L., & Rodriguez-Sanchez, A. (2024). A metabolomics perspective on the effect of environmental micro and nanoplastics on living organisms: A review. *Science of The Total Environment*, *932*, 172915. https://doi.org/10.1016/j.scitotenv.2024.172915

Zhang, Y. T., Chen, H., He, S., Wang, F., Liu, Y., Chen, M., Yao, G., Huang, Y., Chen, R., Xie, L., & Mu, J. (2021). Subchronic toxicity of dietary sulfamethazine and nanoplastics in marine medaka (Oryzias melastigma): Insights from the gut microbiota and intestinal oxidative status. *Ecotoxicology and Environmental Safety*, *226*, 112820. https://doi.org/10.1016/j.ecoenv.2021.112820

Zhao, Y., Qiao, R., Zhang, S., & Wang, G. (2021). Metabolomic profiling reveals the intestinal toxicity of different length of microplastic fibers on zebrafish (Danio rerio). *Journal of Hazardous Materials*, *403*, 123663. https://doi.org/10.1016/j.jhazmat.2020.123663

Zhou, R., Zhou, D., Yang, S., Shi, Z., Pan, H., Jin, Q., & Ding, Z. (2023). Neurotoxicity of polystyrene nanoplastics with different particle sizes at environment-related concentrations on early zebrafish embryos. *Science of The Total Environment*, *872*, 162096. https://doi.org/10.1016/j.scitotenv.2023.162096

Zhou, Y., Gui, L., Wei, W., Xu, E. G., Zhou, W., Sokolova, I. M., Li, M., & Wang, Y. (2023). Low particle concentrations of nanoplastics impair the gut health of medaka. *Aquatic Toxicology*, *256*, 106422. https://doi.org/10.1016/j.aquatox.2023.106422

Zhou, Y., Jin, Q., Xu, H., Wang, Y., & Li, M. (2023). Chronic nanoplastic exposure induced oxidative and immune stress in medaka gonad. *Science of The Total Environment*, *869*, 161838. https://doi.org/10.1016/j.scitotenv.2023.161838

Zhou, Y., Zhao, L., Xu, H., Xu, E. G., Li, M., & Wang, Y. (2022). Long-Term Exposure to Polystyrene Nanoplastics Impairs the Liver Health of Medaka. *Water*, *14*(17), 2767. https://doi.org/10.3390/w14172767